Environmental influences on the particle sizes of purified κ-casein: metal effect

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Abstract

Kappa-casein as purified from bovine milk exhibits a rather unique disulfide bonding pattern as revealed by SDS-PAGE. The disulfide bonded caseins present, range from dimer to octamer and above and preparations contain about 10% monomer. All of these heterogeneous polymers, however, self-associate into nearly spherical uniform particles with an average radius of 8.9 nm as revealed by negatively stained transmission electron micrographs. Evidence is presented that multivalent cations play a role in the stabilization of these spherical particles. Treatment with EDTA causes disruption of the κ-casein particles and leads to a broader size distribution as judged by electron microscopy, dynamic light scattering and analytical ultracentrifugation. The size and shape of the particles are in accord with earlier proposed 3D models for κ-casein, that actually predicted participation of divalent cations in the structure. Published by Elsevier Science Ltd.

Keywords: Casein interactions; Protein structure

1. Introduction

The major proteins of milk, the caseins, form a unique colloidal complex with inorganic phosphate and calcium termed the casein micelle (Farrell, 1988; Holt, 1992; Schmidt, 1982). In bovine milk, this colloidal complex is thought to be composed of submicelles which in turn are aggregates of three calcium-insoluble proteins (α_{s1}-, α_{s2}- and β-casein) and one calcium-soluble protein κ-casein. It is the latter protein which imparts colloidal stability to the system (Holt, 1992; Swaisgood, 1992); indeed, hydrolysis of κ-casein, in the complex, by proteolytic enzymes triggers a cascade leading to coagulation during digestion and release of the nutritionally beneficial calcium and phosphate.

As purified from milk, κ-casein occurs as a high molecular weight complex with a rather unique disulfide bonding pattern as revealed by SDS-PAGE under non-reducing conditions (Groves, Dower & Farrell Jr., 1991). This paper investigates the overall polymer size distribution of purified κ-casein as determined by electron microscopy and correlates these observations with predictive molecular models for κ-casein (Kumosinski, Brown & Farrell, 1993). These comparisons and subsequent experimental studies led to the conclusion that metal ions may play a role in stabilizing the structure of purified κ-caseins.

2. Materials and methods

Casein was isolated from the skim milk of a single cow by precipitation at pH 4.5–4.6. It was recovered by lyophilization after washing with water.

Kappa-casein was isolated from whole casein following the method of McKenzie and Wake (1961) and characterized as previously described by Farrell et al. (1996).

A Perkin–Elmer Model 1100 atomic absorption analyzer unit was used for this study.

Five milligrams of each κ-casein sample were digested in 5% nitric acid for 15 h on a steam bath, after which samples were analyzed for iron and calcium.

Samples of the κ-casein were prepared for electron microscopy and analyzed as previously described (Farrell et al., 1996).

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For the molecular modelling work, a full description of the concepts behind the use of the molecular force fields (including relevant equations) was given in previous communications (Kumosinski & Farrell, 1994; Kumosinski, King & Farrell, 1994). A Tripos force field was used to achieve minimization of the calcium-caseinate complex (because of its ability to handle non-protein atoms without having to define a new library entry for calcium 'residues'). A non-bonded cut-off of 0.8 nm was used.

Dynamic light scattering was measured with a Malvern System Model 4700c equipped with a 256 channel correlator as previously described (Farrell et al., 1996).

FTIR measurements were made on the κ-caseins at 25°C in water using a Nicolet 740 FTIR spectrometer. Data accumulation and analysis, as well as sample compartment modifications have been described (Kumosinski & Unruh, 1996).

For analytical ultracentrifugation, the κ-casein samples were dissolved at pH 6.75 in 35 mM PIPES, 80 mM KCl at concentrations of 1.0–3.0 mg ml^{-1}. The samples and solvents were filtered with a Waters (Milford, MA) HVLP 0.45 μm membrane filter. For κ-casein, Phast gel electrophoresis in SDS showed a nearly identical pattern of protein component before and after filtration; less than 1% of the material was retained on the filter as ascertained by UV spectroscopy. Sedimentation velocity experiments were performed in a Beckman Optima XL-A (Palo Alto, CA) analytical ultracentrifuge at a speed of 22,500 rpm at 25°C. A 12 mm charcoal-epon dual channel centerpiece was used with quartz windows in a wide aperture window holder. Data were collected at 280 or at 292 (samples >1.0 mg ml^{-1}) using the standard XL-A procedure. The absorption versus distance plots were analyzed directly for sedimentation constants using the program TRANSPORT or SECOND MOMENT which are part of the Optima XL-A data analysis software. Apparent sedimentation coefficient distributions (i.e., uncorrected for the effects of diffusion), $g^*(s)$ were calculated from $(\partial c/\partial t)_r$ as:

$$g^*(s)_t = \left(\frac{\partial c}{\partial t}\right)_{\text{corr}}\left(\frac{1}{c_0}\right)\left(\frac{\omega^2 t^2}{\ln (r_m/r)}\right)\left(\frac{r}{r_m}\right)^2, \quad (1)$$

where s is the sedimentation coefficient, ω is the angular velocity of the rotor, c_0 is the initial concentration, r is the radius, r_m is the radius of the meniscus, and t is time. Values of $g^*(s)_t$ obtained this way are identical to those of $g^*(s)$ calculated from the radial derivative to within the roundoff error of the computations (Stafford, 1992).

For sedimentation equilibrium studies, absorption versus radius squared plots were analyzed directly for weight average molecular weight using the program IDEAL 1 or ASSOC4 which are part of the Optima XL-A data analysis software.

The ASSOC4 model is for a system with up to four species; here the best fits were obtained by assuming that κ-casein exists as a covalently and non-covalently bound 'monomer' that self-associates to polymers of higher order. The equation used is

$$A_r = \exp[\ln A_0 + HM(x^2 - x_0^2)] + \exp[N_2 \ln A_0 + \ln(K_{A_2}) + N_2 HM(x^2 - x_0^2)] + E, \quad (2)$$

where A_r is the total absorbance of all species at radius x, A_0 is the absorbance of the monomer species at reference radius x_0, H is the constant $(1 - \nabla\rho)\omega^2/2RT$, M is the 'monomer' molecular weight (covalent and non-covalent polymers), x_0 is the reference radius, N_2 is the stoichiometry for species 2 (number of 'monomers'), K_{A_2} is the association constant for the monomer-nmer equilibrium of species 2.

3. Results and discussion

When bovine κ-casein is subjected to SDS-PAGE in the absence of reducing agents, distinct polymers (at least 8–10) of orderly increasing size are observed (Groves et al., 1991). On electrophoresis of κ-casein after reduction with 2-mercaptoethanol, the polymers disappear resulting in a single band representing the κ-casein monomer. Rasmussen, Højrup and Petersen (1992) demonstrated that these polymers represent a random cross linking pattern using all three possible combinations of disulfide bonds (11–11, 88–88 and 88–11).

3.1. Molecular modelling of the κ-casein polymers

Previous studies from this laboratory have generated a three-dimensional molecular model for κ-casein monomer (Kumosinski et al., 1993). This monomer model was used to assemble disulfide linked tetramers with an asymmetric arrangement of disulfides (11–88, 11–11 and 88–11). The calculated radius of gyration for this particle is 4.7 nm which converts to a hydrodynamic radius of 6.1 nm, somewhat smaller than the experimentally observed particles, and smaller than the experimental molecular weight (150,000–600,000 Da) (Swaisgood, Brunner & Lilevik, 1964). To simulate more fully the experimental data, two tetrameric structures similar to Fig. 1A were docked at various angles relative to each other and then energy minimized. The apparent best fit, based on minimized energy was about a 90° angle. A small degree of energy reduction (-237 kcal mol^{-1}) occurred. The calculated hydrodynamic radius for this molecule was 8.0 nm, which is more in agreement with the experimental data (Fig. 1A). On observation of the first octamer molecular model, small pockets of net negative charge on the adjacent tetramers were observed and through charge repulsions they could deter docking. Four Ca^{2+} ions were added to the octamer, 2 each, to

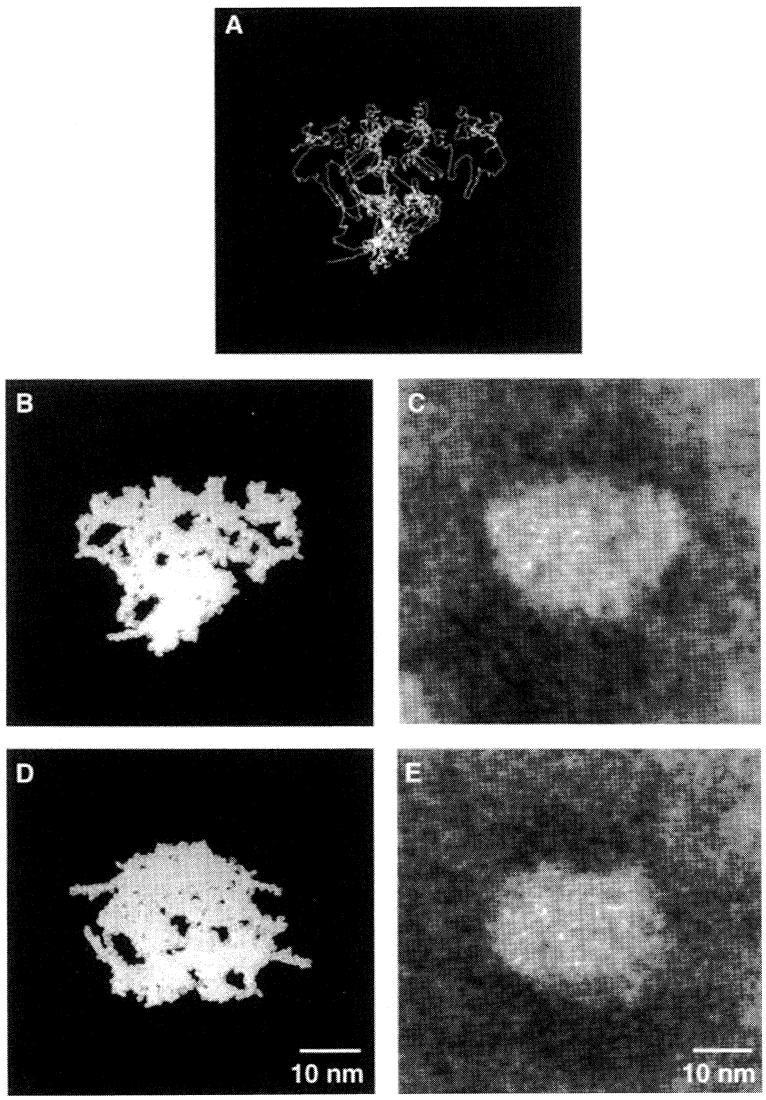

Fig. 1. Comparison of κ-casein octamer 3D model after 20 ps of molecular dynamics with photographic enlargements of image enhanced representations of κ-casein. (TEM bar = 10 nm; molecular model bar = 10 nm). (A) Top: backbone structure for octamer of κ-casein; (B) left: calculated van der Waals surface of (A); (C) right: photographic enlargement of image enhanced EM representation of a κ-casein particle; (D) rotation of 90° about *x* axis of (B) van der Waals model and photographic enlargement of image enhanced EM representation of a κ-casein particle (E) showing a comparable structure. Bar = 10 nm.

bridge Glu-151 to Glu-151 and Glu-12 to Glu-12. Addition of four Ca^{2+} ions to the model reduced the total energy of the system by 1441 kcal mol^{-1} as measured during 20 ps molecular dynamics trajectories at 50K using the Tripos force field. These molecular dynamics calculations yielded a radius of gyration of 5.4 nm or a hydrodynamic radius of 7.0 nm. Thus the divalent cations could lend stability to the κ-casein particles. It should be kept in mind that the model systems employed here do not contain water, and thus the energies reported are relative to each other and have only qualitative significance. The calculated van der Waals energy minimized octamer is compared with photographically enhanced electron microscopy images (Fig. 1B–E).

3.2. Divalent metal ion contents of the purified κ-casein and effect of EDTA

The McKenzie–Wake method (1961) of κ-casein preparation involves a number of classical protein isolation techniques such as acid, salt and alcohol fractionation. The κ-casein preparations were subjected to atomic absorption analysis and were found to contain two major cations: calcium and iron. For three preparations, 0.22 mol of calcium and 0.19 mol of iron were found per mole of protein monomer with about 20% variance from preparation to preparation. This equals on average 1.8 mol of Ca^{2+}, and 1.5 mol of Fe^{2+} per octamer of κ-casein. It is possible, as suggested by analysis of the

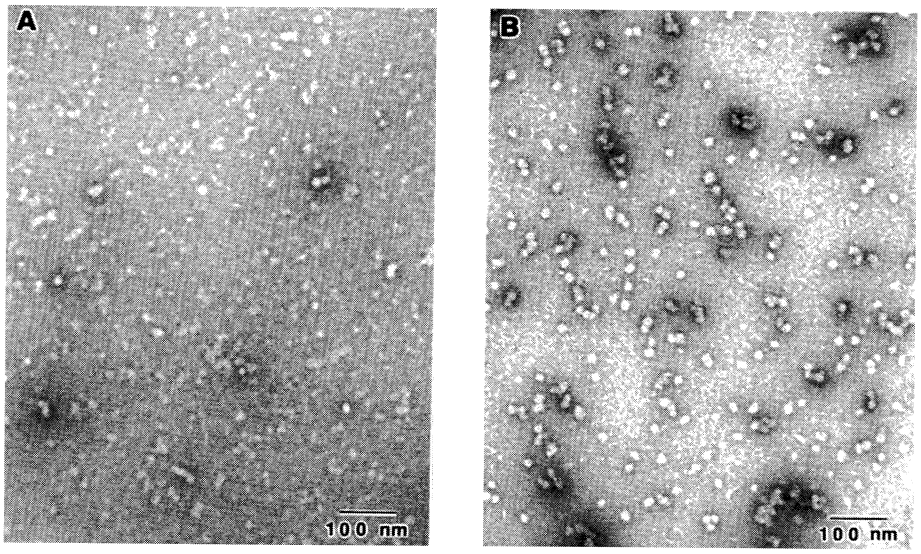

Fig. 2. Comparison of size and shape of κ-casein particles in (A) PIPES-EDTA and (B) PIPES-KCl as determined by transmission electron microscopy.

molecular models, that these cations play a role in the stabilization of the κ-casein complexes, observed in Fig. 2, and as measured by gel permeation chromatography (Pepper & Farrell, 1982).

3.3. *Effects of EDTA on electron microscopy and dynamic light scattering of κ-casein*

To test the hypothesis that metal ions effect κ-casein's physical state, the protein was dissolved in the PIPES buffer at pH 6.75 either with 80 mM KCl or with 14 mM trisodium EDTA to replace the KCl while maintaining constant ionic strength. Transmission electron microscopy was carried out in parallel on both KCl and EDTA samples. The κ-casein particles were visualized by negative staining. The net effect of the EDTA was to partially disrupt the uniform distribution seen in Fig. 2B. Many of the κ-casein particles are seen as diffuse and poorly organized or partially aggregated and the κ-particles seem to be flatter in EDTA (Fig. 2A) and their uniform character and nature in KCl (Fig. 2B) are dramatically altered.

To characterize more quantitatively this shift in size distribution, κ-casein was dissolved in PIPES KCl or EDTA as described above at concentrations of 4 $g\,l^{-1}$. These samples were analyzed for particle size at 30, 60, 90 and 120° by dynamic light scattering. The treatment with EDTA yielded an increase in overall size, accompanied by a shift to a somewhat less asymmetrical percentage weight distribution (Fig. 3). This weight percentage distribution could be influenced by the larger sizes generated by EDTA; for each angle, the Z average diameters increased more dramatically than the mean diameters. Overall, the average weight % diameter shifted from 24.9

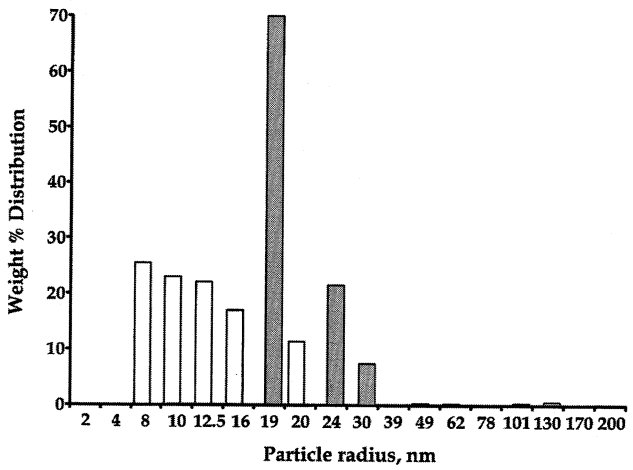

Fig. 3. Comparison of angle dependent weight % particle size distributions for κ-casein in PIPES-KCl (clear) and EDTA (shaded) as determined by dynamic light scattering.

to 42.9 nm. The number average radius shifted from 9.6 ± 2.5 nm in KCl to 19.9 ± 2.4 nm in EDTA; similar results were given by a second preparation. Thus the data from both electron microscopy and light scattering argue for altered particle size and shape on treatment of purified κ-casein with EDTA. This indicates that the metal ions which are either normally associated with κ-casein or become associated with it during purification play a rather distinct role in the particle size distribution observed for the purified protein. Because of the variance in the type and amounts of ions present, it is likely that the binding represents a series of multiple equilibria, rather than specific binding sites.

3.4. Effects of EDTA on secondary structural elements of κ-casein as determined by FTIR

FTIR measurements were made on purified κ-casein dissolved in water in PIPES-KCl or EDTA. Deconvolution of the amide I and II bands and summation of the areas attributable to prominent structural elements yielded the results of Table 1. The results for κ-casein in KCl are in accord with earlier secondary structural analysis of casein by Raman spectroscopy (Byler, Farrell & Susi, 1988). The repeatability of the FTIR methodology as shown in Table 1 is similar to that found for globular proteins (Kumosinski & Unruh, 1996). Thus changes in extended (β-) structures and in α-helical or 'bent-sheet' structures are most likely significant; there appears to be a loss of the former and a gain in the latter on treatment with EDTA. While these are not dramatic, some secondary structural changes accompany the overall alteration of κ-casein particles by EDTA. It is interesting to note that the β-sheet (extended) structures which are prominent in the interactions used to dock the 3D models (Fig. 1) are decreased in the EDTA treated samples and more bent-sheet (or possibly helical) elements are formed.

3.5. Effects of EDTA on sedimentation of κ-casein

Sedimentation velocity studies were carried out at 22,500 rpm and 25°C to assess the effects of EDTA on purified κ-casein. The sedimentation constants calculated either by second moment analysis or by the standard transport method were not significantly different at 24.4 ± 1.2 S_{25}. However, dynamic light scattering results suggested greater polydispersity in EDTA. By analyzing the time derivative of sedimentation, an apparent sedimentation coefficient distribution, $g^*(s)$, can be derived (Stafford, 1992). The overall distribution for the EDTA sample appears broader and flatter than the KCl control (Fig. 4A). Second derivative analysis of the two samples shows that the KCl sample centers on 20.3s^* with major peaks at 18.20 and 22s^*, while the EDTA profile is broader and contains major components ranging from 12 to 26s^*. This analysis confirms the results from dynamic light scattering.

In sedimentation equilibrium experiments, the weight average molecular weight of the EDTA treated κ-casein decreased to $(0.892 \pm 0.062) \times 10^6$ from a value of $(1.18 \pm 0.13) \times 10^6$ Da for the KCl sample. The fits of these data to the IDEAL 1 model were poor indicating pronounced self-association for both samples at higher κ-casein concentrations. Fitting the data to an associated model (Eq. (2)) produced better fits. This model assumes a monomer (M) and that a number of monomers (N) self-associate to a polymer with a constant, K_A l g^{-1}. For the 'native' κ-casein the results were best fit by a particle (M) of mol. wt. 650,000 polymerizing with a K_A of 5 ± 2 and $N = 5$ (average 3 experiments). For the EDTA treated sample a particle of 540,000 could polymerize with $N = 8$ and a K_A of 22 ± 5. Such values for the EDTA treated sample argue for decreased weight average molecular weight and a greater overall polydispersity, because of the larger K_A and N values. These data are also in accord with other physical observations of the effect of EDTA on κ-casein.

Table 1
FTIR estimations of the secondary structural elements of κ-casein

Structural element	KCl%	EDTA%
Turns	25.2 ± 1.8	21.2 ± 1.6
Helix[a]	16.7 ± 2.1	24.5 ± 2.9
Extended[b]	35.2 ± 3.2	28.0 ± 2.5
Irregular	23.0 ± 3.9	26.2 ± 4.4

[a]Includes 3–10 helix and 'bent-sheet' structures.
[b]Includes β-sheet.

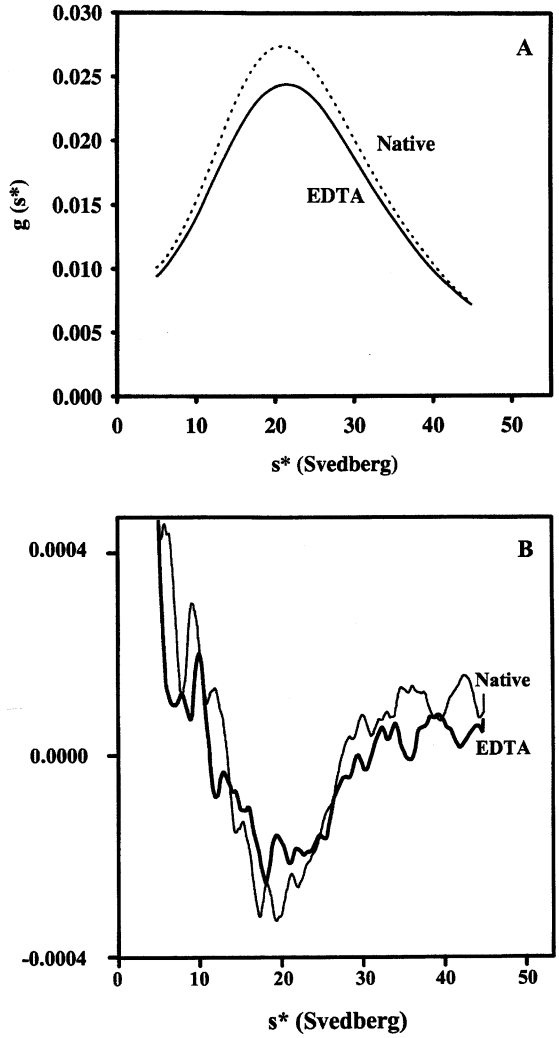

Fig. 4. Comparison of the sedimentation distribution $g^*(s)$ for κ-casein in PIPES-KCl and EDTA. Smoothed function (A), is described in Eq. (1), and its second derivative (B).

4. General conclusions

Kappa-casein as purified from bovine milk exists as rather uniform spherical particles of 8.9 nm radius. This is at odds with some physico-chemical data for κ-casein (Table 2). However, the data presented here are for unfractionated κ-casein, while much of the other data (de Kruif & May, 1991; Thurn, Blanchard & Niki, 1987 and Vreeman, Visser, Slangen & Van Riel, 1986) were collected on samples which were reduced, purified on DEAE media in urea to remove glycosylated forms, dialyzed, lyophilized (Vreeman, Brinkhaus & Van der Spek, 1981) and then redissolved and reduced to varying extents (Table 2). The process of further isolation by use of urea and reducing agents may yield differently sized particles. In this study the values for 'native' bovine κ-casein particles appear to fall between those found for completely and fully reduced samples (Table 2); for example the weight average molecular weight for κ-casein was found to be $(1.18 \pm 0.13) \times 10^6$ Da.

An overall view of κ-casein as purified from bovine milk is that the protein contains a series of disulfide bonded polymers, ranging from monomers to octamers and above (Groves et al., 1991). These polymers are most likely distributed among three possible arrangements of disulfides (11–88, 88–88, and 11–11) according to Rasmussen et al. (1992). The apparent heterogeneity of the individual κ-casein chains, however, is overcome through protein–protein interactions which yield rather uniform particles with radii of 8.9 nm as revealed by electron microscopy or 9.6 nm (number average) by dynamic light scattering. The shape and size of the κ-casein particles may be mediated in part by metal cations such as calcium and iron. However, no specificity for these ions can be implied from the data. Removal of multivalent cations by EDTA yields more heterogeneous particles which could be anticipated by the SDS-PAGE data. Finally, the previous models for κ-casein (Kumosinski et al., 1993) suggested possible internal and external sites for divalent cation binding to enhance closer approach and yield the more compact particles observed by electron microscopy in KCl. Subsequent measurements in EDTA support this notion.

In its native environment within the colloidal casein complex, κ-casein most likely occurs in close association with the more phosphorylated caseins (Farrell & Thompson, 1988). During the isolation procedure such heteropolymeric interactions are most likely replaced by homopolymeric associations which could lead to sequestration of multivalent ions. It may be speculated that the multivalent cations replace the interactions between cationic (Lys, Arg) groups on the caseins and κ-casein. On the other hand, the binding of divalent cations could be important during the process of casein micelle maturation. Charge neutralization of, e.g. residues 147–151 on κ-casein could allow closer approach of a number of κ-casein monomers on the micelle surface. Such κ–κ-interactions would in turn promote κ-casein 'caps', steering the monomers toward the formation of κ-casein polymers through subsequent disulfide binding. This would be analogous to the lateral movement of cadherins or cell adhesion molecules on cell surfaces, which is thought to be mediated in part by Ca^{2+} (Takeichi, 1990). Thus both hydrophobic and ionic interactions could play a role in the protein–protein interactions during the assembly of the casein micelle within Golgi vesicles.

Table 2
Summary of physical data on κ-caseins

	MW	Radius (nm)[a]	Method	Reduction
Vreeman et al. (1986)	600,000	11.1[b]	Sedimentation	1 week, 2-ME[c]
Slattery and Evard (1973)	600,000	11.2[b]	Sedimentation	1 h, 40 mM DTT
de Kruif and May (1991)		14.7[b]	SANS[e]	5 mM DTT
Pepper and Farrell (1982)		9.4[d]	GPC[f]	None
Thurn et al. (1987)	2,000,000	7.0[b,g]	SANS[e]	None
This study		8.9[d]	EM[h]	None
		9.6[d]	DLS[i]	None
	1,180,000	—	Sedimentation	None

[a]Radius type varies with method.
[b]DEAE purified K–I casein.
[c]2-Mercaptoethanol.
[d]Whole κ-casein.
[e]Small-angle neutron/scattering.
[f]Gel permeation chromatography.
[g]Internal 'submicellar' particle of larger aggregate.
[h]Electron microscopy.
[i]Dynamic light scattering, number average.

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